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Campylobacter jejuni Gene Expression in the Chick Cecum: Evidence for Adaptation to a Low-Oxygen Environment

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Transcriptional profiling of *Campylobacter jejuni* during colonization of the chick cecum identified 59 genes that were differentially expressed in vivo compared with the genes in vitro. The data suggest that *C. jejuni* regulates electron transport and central metabolic pathways to alter its physiological state during establishment in the chick cecum.

Campylobacter jejuni is the most common cause of bacterial gastroenteritis in humans in the developed world (1), and infections have been closely linked to the consumption of contaminated poultry products and poor handling of raw meats in the kitchen (17, 23). Interventions throughout the food-processing chain have been rather ineffective in reducing incidents of food poisoning due to this bacterium (2, 26, 42). One strategy for reducing contamination of finished poultry products is to reduce the levels of *C. jejuni* organisms being carried in chickens on the farm and at the point of slaughter. Attempts to achieve this have been made, such as increased biosecurity at the farm level and competitive exclusion, but these have had little impact on the colonization of flocks (24). Thus, other intervention strategies need to be explored to achieve a reduction in flock colonization. A major obstacle to this is that very little is known about the biochemistry and genetics of the interaction of *C. jejuni* with its chicken host. To redress this lack of knowledge, a whole-genome microarray derived from *C. jejuni* NCTC11168 was used to investigate bacterial genes expressed in vivo in the chicken cecum relative to genes expressed when the bacteria are grown in the laboratory.

Chick colonization protocol and sample collection. For these studies, commercial Lohmann brown-egg layers were supplied in ovo (Poultry First, Woodhall Spa, United Kingdom) and hatched in prefumigated incubators. The chicks were used within 10 h of hatching to avoid the development of gut flora. Birds were given an oral inoculation of 0.1 ml of 10^9 CFU of *C. jejuni* 11168H (16, 18), which had been grown for 12 h in Mueller-Hinton (MH) broth (Oxoid, Basingstoke, United Kingdom) under microaerophilic conditions (10% [vol/vol] O_2, 5% [vol/vol] CO_2, and 85% [vol/vol] NO_2). At 12 h postinfection birds were killed individually followed by immediate removal of the cecal contents into Tri reagent (Sigma, Poole, United Kingdom). In a single experiment, cecal contents from 90 birds were added directly into Tri reagent and pooled, and then total RNA was extracted by using the manufacturer's method, starting with 1 volume cecal contents to 10 volumes reagent. Total RNA was treated with DNase I and cleaned using a Midi RNeasy column (QIAGEN, Crawley, United Kingdom). Purified RNA was further concentrated by an ethanol precipitation overnight at −80°C and redissolved in diethyl pyrocarbonate-treated water to a final concentration of 1 μg RNA/μl. Experiments were performed in triplicate. Cecal contents from five randomly picked birds from each group were assessed for colonization by both *Campylobacter* and other bacteria present in the gut. *Campylobacter* levels were assessed by plating serial dilutions of cecal contents onto blood-free *Campylobacter*-selective agar (Oxoid) containing CCDA and CM739 selective supplements (Oxoid). Birds were colonized at 12 h postinfection (data not shown). Gut flora assessment was carried out on nonselective Luria-Bertani agar and MacConkey agar (SR155; Oxoid) to distinguish coliforms. Plates were incubated under both aerobic and microaerophilic conditions. In all three experiments, chicks tested for bacterial contamination were negative for contamination. Total RNA was also extracted from *C. jejuni*-free chicks to determine whether cecal contents alone would result in microarray cross-hybridization or background; none was observed (results not shown). Total RNA was extracted from two in vitro controls comprising *C. jejuni* isolates grown on MH agar plates for 24 h and in MH broth cultures for 12 h under microaerophilic conditions. The microarray results showed that minor differences in gene expression were observed between the two in vitro controls, although more consistent data were obtained when total RNA from bacteria grown on agar was used (http://bugs.sghms.ac.uk/). To determine whether RNA samples contained residual genomic DNA, a standard PCR was carried out to assess the ability to amplify a 200-bp gene fragment corresponding to restriction modification genes. No product was detected from any of the RNA samples tested (data not shown).

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TABLE 1. Primers used to amplify the internal fragments of the target genes and probes end labeled with 6-carboxyfluorescein and 6-carboxy tetramethylrhodamine

Gene	Primer		Probe[a]	PCR product (bp)
	Forward	Antisense		
16S rRNA	5′-ccagcagccgcggtaat-3′	5′-gccctttacgcccagtgat-3′	5′-ccgagtaacgcttgcaccctccg-3′*	60
Cj0088	5′-gcagcagtttcagcacttttgt-3′	5′-tctagtcgttcctgtatcatccattt-3′	5′-cttccaacttatccgactttggttaggtgcagtg-3′	83
Cj0087	5′-agccaaagaggcaatgaacact-3′	5′-tttcatcaatttgttctttgcttaaaa-3′	5′-tctttcaagcgcaatatcagccactcttttt-3′*	135
Cj0239c	5′-tgtggaatttgctatgcgtgata-3′	5′-gccataaccgaacagtgcatt-3′	5′-tccagaaactccagctgttccacctcaaa-3′	74
Cj0410	5′-cgtgaaaaaatggatgcagatc-3′	5′-tggcactccattaatcatcattg-3′	5′-cgcaaatccctgcacgacaaacaaag-3′*	89
Cj0437	5′-ttgcaaatggtggaactcttattaca-3′	5′-acgctcaccacgattatttaaaagatat-3′	5′-ttcaccgcgcgccgctt-3′*	76
Cj0671	5′-tggacttatgctgtaatgcttctttta-3′	5′-gccaaaggaacaaaagctgaa-3′	5′-ttcaaaatttgtaaactctcaagcagcggcta-3′	82
Cj1192c	5′-tgccgccttttcagcaat-3′	5′-aagacatgaaatcagcattactaaaagtaaa-3′	5′-catagctcaatacggaattggctctttgatca-3′	89
Cj1228c	5′-tgatttaatggaaggagatgttgttt-3′	5′-aagcagatattatcccacttgtaacacta-3′	5′-aaccaactccaaaaggatttccaagtgca-3′*	85
Cj1357	5′-aaattattaaaattcaacatccagaaagtg-3′	5′-acaatccacgcaacttactccat-3′	5′-tgcagcatgcacaccgccactataaa-3′*	82
Cj1576c	5′-tgggtattagtggtgcggtttt-3′	5′-gtgctttttggctaaagaatgg-3′	5′-agctcccgttacaataccatgtgcaaaca-3′*	137
Cj1614	5′-gcaaaaatacagcagtggctat-3′	5′-gggcgattgatttgtgtgatatta-3′	5′-caagctcaaagcgatccaacccaaaaa-3′	96

[a] *, probe designed with reverse complement.

Microarray hybridizations and data analysis. Hybridization probes were generated from 5 μg of total RNA extracted directly from chick cecal contents. Total RNA was mixed with 3 μg/μl random primers (Invitrogen, Paisley, United Kingdom), heat denatured, snap cooled on ice, and then reverse transcribed to cDNA to incorporate the fluorescent analogs. Five microliters of 5× first-strand buffer, 2.5 μl dithiothreitol (100 mM), 2.3 μl dNTPs (5 mM A/G/T/TP, 2 mM dCTP; Amersham Biosciences, Chalfont St. Giles, United Kingdom), 1.7 μl Cy3 or Cy5 (Amersham Biosciences, Chalfont St. Giles, United Kingdom), and 2.5 μl SuperScript II (200 U/μl; Invitrogen, Paisley, United Kingdom) were added. Samples were incubated at 25°C in the dark for 10 min and then at 42°C in the dark for 90 min. The corresponding in vivo and in vitro cDNA reaction mixtures labeled with Cy3 and Cy5, respectively, were combined and purified by use of a MinElute column procedure (QIAGEN). Prior to hybridization, 4.6 μl filtered 20× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and 3.5 μl filtered 2% (wt/vol) sodium dodecyl sulfate were added to 14.9 μl of labeled cDNA sample, heated for 2 min, and allowed to cool before application to the microarray slide. *C. jejuni* microarray slides were hybridized overnight with a single reaction, and a minimum of two hybridizations was performed for each in vivo sample. Slides were postprint processed, prehybridized, and hybridized as described previously (15, 16). Slides were scanned with an Affymetrix 428 scanner (MWG Biotech, Milton Keynes, United Kingdom) following the manufacturer's instructions. ImaGene 5.5 software (BioDiscovery, Marina Del Ray, CA) was used for spot quantification. Each quantified spot was filtered by requiring that the signal intensity/background intensity ratio was >2 using the local median background subtraction method. GeneSpring 6.1 software (Silicon Genetics, Redwood City, CA) was used for further data analysis. Background-subtracted, normalized signal intensity ratios (Cy5/Cy3) for each gene were calculated. Genes were identified as differentially regulated (i) if there was a twofold or greater change in expression level, (ii) if the signal intensity of each spot was greater than two times the standard deviation of the background, (iii) and if *t* test results had a probability *P* value of ≤0.05. In this study, differentially expressed genes were filtered by selecting a value of >2.0-fold change in gene expression. This is a greater stringency than has been used in some other studies (3, 35), as we wanted to ensure that data indicating differentially expressed genes were likely to be biologically relevant.

The genes which showed differential expression between in vivo and in vitro conditions and conformed to the statistical analysis filters are shown in Tables 2 and 3.

Quantitative real-time RT-PCR. Real-time quantitative reverse transcription-PCR (qRT-PCR) was used to confirm the gene expression ratios identified by microarray analysis of 12 genes, which were either differentially regulated or constitutively expressed. Primers and fluoroprobes were designed with Primer Express software (PE Applied Biosystems) (Table 1) and purchased from Sigma Genosys Ltd. One-step qRT-PCRs were performed in triplicate by using a mix of 2 ng/μl DNase-treated total RNA, gene-specific primers (50 nM), and probes (100 mM) plus reverse transcriptase qPCR master mix (RT-QPRT-032X; Eurogenetic, EGT Group, Belgium). The concentrations of primer and template in each reaction mixture were determined by the construction of a standard curve starting with 200 ng total RNA and 500 nM primer and using 10-fold dilutions from 10^{-1} to 10^{-5} (data not shown). Three total RNA samples were analyzed in triplicate PCRs, and therefore, nine replicate values were used to generate the standard curves. Amplification and detection of specific primers were performed using the ABI Prism 7700 sequence detection system (PE Applied Biosystems, Warrington, United Kingdom). The cycle parameters were as follows: an initial reverse transcription step for 30 min at 48°C, and then GoldStar DNA polymerase was activated for 10 min at 95°C followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. The results were expressed in terms of threshold cycle value, the cycle at which the change in the reporter dye passes a significance threshold (data not shown). The changes in gene expression (*n*-fold) calculated from the qRT-PCR data were converted to $\log_2$ values and plotted against the changes calculated from the microarray data, which had also been $\log_2$ converted (Fig. 1). The real-time qRT-PCR standard curves showed the slope of the 16S rRNA control gene was 3.28, and the 11 other genes had slopes which ranged from 3.01 to 3.63 (data not shown). The best-fit linear regression line was 1.04, and the regression analysis ($r^2 = 0.82$) showed that a strong correlation was found between qRT-PCR and the microarray technology.

TABLE 2. *C. jejuni* genes up-regulated during chick colonization[a]

Gene class	Gene designation	Function	Change in expression level (*n*-fold)	*P* value
Small molecule metabolism				
Degradation	Cj1624c	L-Serine dehydratase, *sdaA*	3.10	1.78×10^{-6}
Energy metabolism				
Tricarboxylic acid cycle	Cj0437	Succinate dehydrogenase, *sdhA*	32.11	1.23×10^{-2}
	Cj0438	Succinate dehydrogenase, *sdhB*	31.25	>0.05
	Cj0439	Succinate dehydrogenase, *sdhC*	15.65	9.18×10^{-4}
	Cj0409	Putative fumarate reductase, *frdA*	2.51	>0.05
	Cj0410	Putative fumarate reductase, *frdB*	2.83	>0.05
Electron transport	Cj1487c	Cytochrome *c* oxidase (*cb*-type), *ccoP*	3.78	2.20×10^{-4}
	Cj1488c	Cytochrome *c* oxidase (*cb*-type), *ccoQ*	3.16	>0.05
	Cj1489c	Cytochrome *c* oxidase (*cb*-type), *ccoO*	4.18	2.37×10^{-5}
	Cj1490c	Cytochrome *c* oxidase (*cb*-type), *ccoN*	4.40	1.30×10^{-6}
	Cj0780	Probable ferredoxin, *napA*	2.67	>0.05
	Cj0781	Probable ferredoxin, *napG*	4.26	7.34×10^{-11}
	Cj0783	Probable ferredoxin, *napB*	2.72	>0.05
	Cj1184c	Ubiquinol cytochrome *c* reductase, *petC*	5.69	5.07×10^{-5}
	Cj1185c	Ubiquinol cytochrome *c* reductase, *petB*	5.43	4.50×10^{-5}
	Cj1186c	Ubiquinol cytochrome *c* reductase, *petA*	4.73	2.98×10^{-6}
	Cj1357c	Putative periplasmic cytochrome *c,* *nrfA*	5.88	1.90×10^{-3}
	Cj1358c	Probable ferredoxin, *napC*	3.77	>0.05
Central intermediary metabolism				
General	Cj0087	Aspartate-ammonia lyase, *aspA*	4.83	>0.05
Sulfur metabolism	Cj0866	Pseudogene, arylsufatase, *ast*	4.06	1.21×10^{-5}
Fatty acid biosynthesis	Cj0328c	Probable, 3 oxoacyl-[acyl] carrier protein syntase, *fabH*	3.09	1.68×10^{-10}
Broad regulatory function (signal transduction)	Cj0448c	Probable MCP protein, putative *acfB*	2.97	1.29×10^{-4}
Macromolecule modification				
Synthesis, modification, and degradation of macromolecules				
Ribosomal protein synthesis and modification	Cj0893c	30S ribosomal protein S1, *rpsA*	3.13	3.21×10^{-7}
Protein translation and modification	Cj0865	Disulfide oxidoreductase, *dsdB*	6.23	2.04×10^{-5}
Cell envelope				
Membranes, lipoproteins, and porins	Cj0561c	Periplasmic protein	4.44	4.46×10^{-3}
	Cj0864	Periplasmic protein	9.81	4.40×10^{-4}
	Cj0952c	Probable membrane protein	3.85	1.35×10^{-5}
Miscellaneous periplasmic proteins	Cj0892c	Periplasmic protein	3.09	4.76×10^{-6}
	Cj0909	Periplasmic protein	2.72	5.57×10^{-5}
	Cj0834c	Probable periplasmic protein	3.38	3.19×10^{-6}
Cell processes				
Transport/binding proteins, cations, carbohydrates, organic acids, and alcohols	Cj1614	Hemin uptake outer membrane protein, *chuA*	40.56	6.25×10^{-4}
	Cj1615	Hemin uptake permease protein, *chuB*	4.07	>0.05
	Cj1619	Probable alpha-ketoglutarate permease, *kgtP*	2.92	9.06×10^{-6}
	Cj0088	Anaerobic C_4 dicarboxylate transporter, *dcuA*	8.63	6.83×10^{-4}
	Cj0671	Anaerobic C_4 dicarboxylate transporter, *dcuB*	19.24	3.25×10^{-3}
Detoxification	Cj0358	Cytochrome peroxidase c551	11.71	9.77×10^{-4}
Miscellaneous	Cj0833c	Probable oxidoreductase	4.83	6.92×10^{-7}

[a] To identify genes showing consistent regulation in all the microarray data, comparing in vivo samples to both in vitro control samples, we applied a signal intensity of more than two times the standard deviation of the background. Further genes were selected on the basis of a change in relative expression level (*n*-fold) of ≥2 and those that showed a *P* value of ≤0.05. Some genes with a *P* value of >0.05 were also included to show complete operons were regulated.

Therefore, the real-time qRT-PCR results validated the microarray data. The slope of the regression line indicates that slightly higher expression changes were measured by qRT-PCR than by microarray analysis (Fig. 1).

C_4-dicarboxylate transport genes show increased transcription in response to limited oxygen. Our microarray data indicate that conditions within chick ceca result in an increase in C_4-dicarboxylate transporter gene transcription. The signal required to regulate *C. jejuni dcuA* and *dcuB* genes is unknown. Studies have shown that in *Escherichia coli*, C_4-dicarboxylate

TABLE 3. *C. jejuni* genes down-regulated during chick colonization[a]

Class	Gene designation	Function	Change in expression level (*n*-fold)	*P* value
Small molecule metabolism				
Energy metabolism	Cj0037c	Possible cytochrome *c*	0.043	1.32×10^{-22}
	Cj0874c	Possible cytochrome	0.280	6.82×10^{-8}
Amino acid biosynthesis	Cj0226	Probable acetylglutamate kinase, *argB*	0.358	2.21×10^{-6}
Biosynthesis of cofactors, prosthetic groups, and carriers	Cj0453	Thiamin biosynthesis protein, *thiC*	0.20	5.87×10^{-6}
	Cj0298c	Probable 3-methyl-2-oxobutanoate hydroxymethyltransferase, *panB*	0.18	2.36×10^{-15}
	Cj0297c	Probable beta alanine ligase, *panC*	0.21	9.32×10^{-9}
Fatty acid biosynthesis	Cj1183c	Probable cyclopropane fatty acid acyl phospholipid synthase, *cfa*	0.224	1.01×10^{-9}
Macromolecule modification				
Synthesis, modification, and degradation of macromolecules				
Ribosomal protein synthesis and modification	Cj0095	50S ribosomal protein, *rpmA*	0.29	9.52×10^{-8}
	Cj0094	50S ribosomal protein, *rplU*	0.21	1.70×10^{-11}
Degradation of macromolecules	Cj1228c	Serine protease, *htrA*	0.21	1.31×10^{-11}
	Cj1360c	Probable proteolysis tag	0.25	1.93×10^{-9}
Cell envelope				
Membranes, lipoproteins, and porins	Cj0629	Possible lipoprotein	0.21	2.23×10^{-6}
	Cj0628	Possible lipoprotein	0.17	1.36×10^{-11}
	Cj0830	Probable integral membrane protein	0.24	3.92×10^{-6}
	Cj1170c	Possible outer membrane protein, omp50	0.28	1.26×10^{-10}
	Cj0987c	Probable integral protein	0.16	8.74×10^{-6}
Miscellaneous periplasmic proteins	Cj1725	Probable periplasmic protein	0.32	3.30×10^{-15}
	Cj0057	Probable periplasmic protein	0.15	3.47×10^{-10}
	Cj1668c	Probable periplasmic protein	0.29	5.81×10^{-12}
	Cj0876c	Possible periplasmic protein	0.28	5.71×10^{-10}
	Cj0425	Putative periplasmic protein	0.34	1.58×10^{-8}
Cell processes				
Transport/binding proteins, cations, anions, carbohydrates, organic acids, and alcohols	Cj0203	Probable transmembrane transport	0.19	6.83×10^{-12}
	Cj0982c	Probable amino acid transporter	0.17	4.22×10^{-10}
	Cj0303c	Probable molybdate binding protein, *modA*	0.22	2.21×10^{-11}
	Cj0025c	Probable transmembrane symporter	0.03	8.38×10^{-24}
Detoxification	Cj0779	Probable thiol peroxidase, *tpx*	0.16	2.28×10^{-9}
Other (conserved hypothetical proteins)	Cj0239c	Probable *nifU* homolog	0.14	2.03×10^{-4}
Miscellaneous	Cj0240c	Probable *nifS* homolog	0.15	2.34×10^{-21}
	Cj0414	Probable oxidoreductase subunit	0.08	7.08×10^{-17}
	Cj0415	Probable oxidoreductase subunit	0.11	3.51×10^{-18}
	Cj0225	Probable acetyltransferase	0.35	1.99×10^{-7}

[a] To identify genes showing consistent regulation in all the microarray data, comparing in vivo samples to both in vitro control samples, we applied a signal intensity of more than two times the standard deviation of the background. Further genes were selected on the basis of a change in relative expression level (*n*-fold) of ≥ 2 and those that showed a *P* value of ≤ 0.05. Some genes with a *P* value of >0.05 were also included to show complete operons were regulated.

utilization genes are up-regulated under anaerobic conditions by the oxygen-sensing Fnr global regulator but are also up-regulated in the presence of C_4-dicarboxylates through a two-component sensor-regulator system, DcuSR (8, 11, 44). A central theme apparent in the pattern of up-regulated genes is a response to limited oxygen supplies in the cecum, as up-regulated genes identified in the microarray screen match genes in other bacteria that are activated at low oxygen tensions or during anaerobiosis. In vitro studies demonstrate that *C. jejuni* cannot grow under strictly anaerobic conditions, but when oxygen supplies are limited and fumarate is added to the culture medium, growth enhancement occurs (32). This suggests that *C. jejuni* can utilize fumarate as a terminal electron acceptor instead of oxygen, supporting increased growth yields. We determined whether limited oxygen and/or the presence of the C_4-dicarboxylates succinate and fumarate leads to up-regulation of C_4-dicarboxylate utilization genes in *C. jejuni*. Changes in expression were determined for four genes, *dcuA* (Cj0088), *dcuB* (Cj0671), *dctA* (Cj1192), and aspartate-ammonia lyase (aspartase) gene *aspA* (Cj0087). Total RNA was extracted

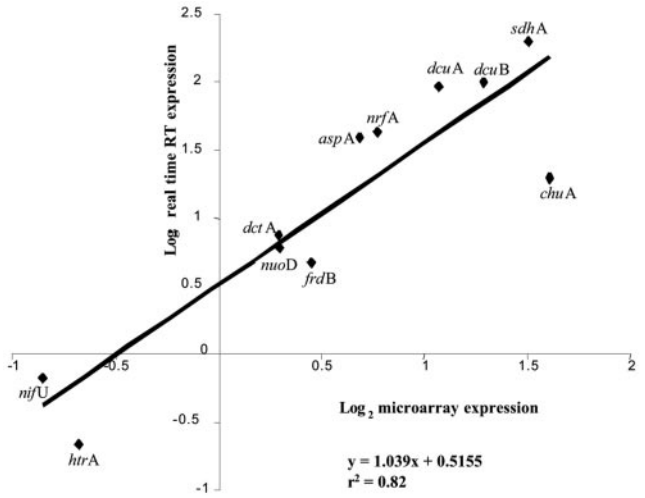

FIG. 1. Correlation between microarray and real-time RT-PCR expression values. $\log_2$-transformed expression values for 11 genes from bacterial total RNA extracted from chick cecal contents in triplicate. The best-fit linear regression line is shown together with the r^2 value and calculated equation for the slope.

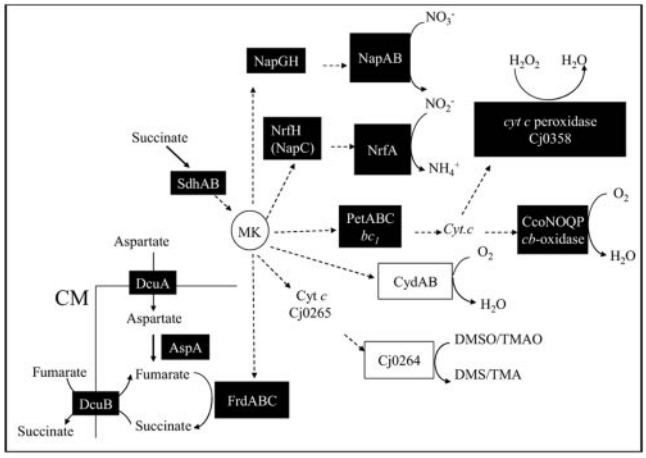

FIG. 3. Possible in vivo electron transport pathways in *C. jejuni*, based on the microarray experiments reported in this study and the deduced electron transport chain structure described in references 25 and 32. Dotted lines show pathways of electron transfer, and solid lines show substrate or electron acceptor transformations. Black boxes represent those proteins or complexes whose gene expression is up-regulated >2-fold in vivo compared to in vitro microaerobic growth, while white boxes denote proteins whose gene expression levels were unchanged. CM (black line), the cytoplasmic membrane; MK, menaquinone pool.

from 11168H cultures grown in microaerophilic and oxygen-limiting conditions (32) in the presence or absence of either fumarate or succinate, and the differential transcriptional change in expression (*n*-fold) was measured by using real-time qRT-PCR (Table 1; Fig. 2). The values (*n*-fold change) were normalized to the data obtained with RNA from cells grown in microaerobic conditions. The data (Fig. 2) indicate that limited oxygen supplies have a greater effect on the expression levels of *dcuA*, *dcuB*, *aspA*, and *dctA* than the presence of the C_4-dicarboxylates fumarate or succinate in microaerobic conditions. It is thus possible that in *C. jejuni*, there are separate, uncharacterized regulatory systems responding to oxygen and C_4-dicarboxylates.

Electron transport and energy metabolism. The electron transport chains in *C. jejuni* are highly branched, and this suggests flexibility in metabolism and physiology allowing adaptation to specific environmental conditions (25, 32). *C. jejuni* encodes a number of reductases for electron acceptors other than oxygen, such as nitrate, nitrite, fumarate, and *S*- or *N*-oxides (e.g., dimethyl sulfoxide and trimethylamine-*N*-oxide [27, 32]). The microarray data show elevated levels of transcription of several of these systems, indicating that nitrate (*nap*), nitrite (*nrf*), and fumarate (*frd*) are all possible substrates available for respiration in vivo (Table 2 and Fig. 3). Previous in vitro studies have shown that *C. jejuni* cannot grow by nitrate respiration under strictly anaerobic conditions (despite such cells being able to reduce nitrate), but under severe oxygen limitation, nitrate reduction can support growth (32). Fumarate can be utilized as an alternative electron acceptor, and Frd activity is increased when *C. jejuni* is grown in oxygen-limited cultures (32, 33). The in vivo availability of fumarate is unknown, but deamination of aspartate by the aspartase encoded by *aspA* may be an important route for its formation, given that *aspA* was up-regulated 4.8-fold in vivo.

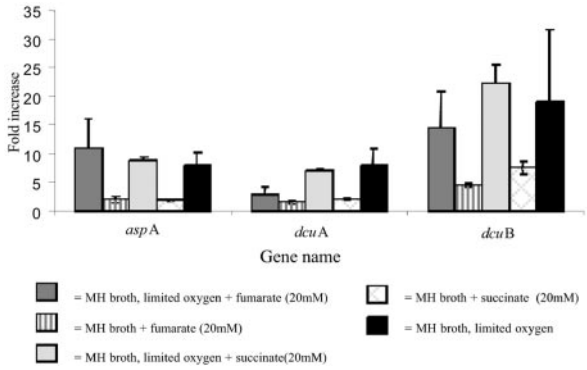

FIG. 2. Levels of *C. jejuni* gene expression in oxygen-limited cultures and in the presence of C_4-dicarboxylates. Gene expression levels of *aspA*, *dcuA*, and *dcuB* were determined from RNA extracted from in vitro microaerobic MH broth cultures and compared to oxygen-limited cultures or oxygen-limited cultures in the presence of succinate or fumarate (20 mM). These experiments were performed in triplicate and standard error bars are shown.

In *C. jejuni*, two different terminal oxidases have been identified: a *bd*-type quinol oxidase (Cyd homologues) and a *cb* (*cbb*$_3$)-type cytochrome *c* oxidase (Cco homologues and Cj1490-Cj1487c) (25, 27). The in vivo microarray data indicate increased expression of both the *cb*-type cytochrome *c* oxidase and the *petABC* operon (Cj1186c-Cj1184c) encoding the proton-translocating cytochrome bc_1 complex, which feeds electrons to the *cb*-oxidase via a *c*-type cytochrome (Fig. 3). In *H. pylori*, the Cco complex has the ability to pump protons and has a K_m for oxygen of 0.04 μM, which indicates a high affinity (37), although as discussed by Kelly et al. (19), the true K_m is likely to be considerably lower. Given the high degree of similarity of Cco subunit amino acid sequences between *H. pylori* and *C. jejuni*, and the known properties of these enzymes, *C. jejuni*

Cco is likely to be able to operate at extremely low oxygen concentrations, in the nM range.

Overall, the pattern of up-regulated gene expression detected by microarray analysis of cells from the chick cecum is consistent with much lower oxygen concentrations in this environment compared with the in vitro microaerobic control. Therefore, it is particularly interesting to see both succinate dehydrogenase (*sdh*) and fumarate reductase (*frd*) up-regulated in vivo. In *E. coli*, succinate dehydrogenase is up-regulated in aerobic conditions and fumarate reductase is up-regulated in anaerobiosis. Both enzymes can function to interconvert succinate and fumarate (5, 34). In *E. coli*, Sdh can function as an effective fumarate reductase in vitro (21, 28), even supporting its anaerobic growth (22). However, it is also possible that succinate dehydrogenase is highly activated in *C. jejuni in* vivo to maintain the oxidative function of the citric-acid cycle under the low oxygen tensions found in the gut. Only the purification and kinetic characterization of these two enzymes will allow their physiological roles to be determined.

Central intermediary metabolism. The carbon sources that *C. jejuni* can utilize in the chicken cecum are unknown, although the most likely source of carbon and nitrogen in vivo is via deamination of amino acids, given that *C. jejuni* is asaccharolytic (41). In previous studies, in vitro experiments have shown that *C. jejuni* can grow in minimal media by using serine, aspartate, glutamate, and proline as carbon sources (20). The microarray data show a >2-fold increase in transcription of serine dehydratase, *sdaA* (Cj1624c), and the putative oxidoreductase Cj0833c, which suggests *C. jejuni* can utilize serine in vivo. SdaA catalyses the deamination of serine to pyruvate and ammonia, both of which can be readily assimilated (40), and while the function of Cj0833c is not obvious, it shares some sequence identity with YdfG in *E. coli* and a serine dehydrogenase from *Agrobacterium tumefaciens*. YdfG requires $NADP^+$ as a coenzyme and can use L-serine as a substrate (10). Interestingly, a *C. jejuni sdaA* mutant does not colonize 3-week-old chickens, suggesting that L-serine is essential for *C. jejuni* growth in vivo (40). This colonization study fits well with the microarray results, showing that *sdaA* is up-regulated in vivo.

In *C. jejuni*, the aspartate-ammonia lyase gene, *aspA* (Cj0087), is downstream of the anaerobic C_4-dicarboxylate transport gene *dcuA* (Cj0088). In *E. coli*, it is likely that these genes are cotranscribed and regulated by similar mechanisms (12). Therefore, in vivo, *C. jejuni* aspartate utilization may also be linked to C_4-dicarboxylate utilization (Fig. 3). Aspartate is deaminated to fumarate by AspA, which can then be metabolized through the citric acid cycle or used as an electron acceptor.

Other relevant up-regulated genes. In *C. jejuni*, iron is fundamental for growth, and to date, nine uptake and transport systems for the acquisition of iron in the environment have been identified (38). From the microarray data, two genes, *chuA* and *chuB*, show 40- and 4-fold increases in transcription, respectively. Previous studies have shown that a *C. jejuni chuA* mutant is unable to grow on hemin as the sole source of iron (31, 39). Although the *chuA* mutant has not been screened through a chicken model, the in vitro data and high up-regulation in vivo strongly suggest that *chuA* might be required for chicken colonization.

Iron plays a role in defense against oxidative stress due to the fact that some oxidative stress protection systems require iron-cofactored-prosthetic groups (36). *C. jejuni* has a number of mechanisms for survival of oxygen stress, including its ability to remove hydrogen peroxide in the cytoplasm via catalase (*katA* [13]). Periplasmic removal is probably mediated by a cytochrome *c* peroxidase, of which there are two possible candidates, Cj0020c and Cj0358. Under in vivo conditions, *C. jejuni* Cj0358 shows a large 12-fold increase in transcription compared to in vitro conditions. This might suggest that Cj0358 is responsible for the removal of hydrogen peroxide from the periplasm and may act as an oxygen-independent terminal hydroperoxidase (25).

It was interesting to see up-regulation of Cj0448c, which shows 29% identity to a predicted protein accessory colonization factor (*acfB*) from *Vibrio cholerae* (9, 29) and 31.9% identity to HP0599 (putative hyaluronate lyase gene *hylB*) from *Helicobacter pylori*. In *V. cholerae*, *acfB* encodes an environmental sensor/signal-transducing protein involved in colonization of the mouse intestine (9). In *C. jejuni*, Cj0448c is highly up-regulated in the chick cecum, and it is the only gene identified in this study to be previously described as a potential virulence factor in another pathogen. A previous colonization study with a mutant of Cj0448c indicates that this gene is not essential for the early stages of colonization (14), but this does not rule out the possibility that it may be involved in persistence of bacteria in the presence of a developed gut flora.

Analysis of down-regulated genes or genes equivalent in expression. It is difficult to interpret how the set of down-regulated genes may play a role in *C. jejuni* chick colonization as many of them only have putative assignments. The down-regulation of *thiC* would indicate that there is a ready source of thiamine in the intestine. An interesting observation was the low transcription of a putative serine protease, *htrA* (Cj1228c) characterized as a stress response protein. In *Salmonella enterica* serovar Typhimurium, *Yersinia pestis*, and *Klebsiella pneumoniae*, *htrA* has been reported as being essential for full virulence (6, 7, 43), although this is not the case for all pathogens, e.g., *Brucella abortus* (30).

The data, as presented, do not highlight important genes expressed at equivalent levels under both conditions. This is due to inherent constraints within the experimental techniques. Therefore, we individually assessed data for several genes previously associated with colonization. The genes *cadF* (45, 46) and *racRS* (4) and genes associated with motility, such as *flaA* (16), were all expressed in vivo at levels equivalent to those seen in vitro.

By focusing on those genes showing increased expression in vivo, we have focused on a subset of genes that may be important in adaptation to the host environment. These results indicate that *C. jejuni* adapts to conditions within the chick cecum by increasing the regulation of specific genes so that this bacterium can efficiently make use of the limited oxygen and nutrient supplies. The presence of multiple respiratory mechanisms and their differential expressions under various conditions may be an advantage for *C. jejuni* in coping with the changes in oxygen availability in vivo (Fig. 3). There is evidence that both oxygen-dependent and oxygen-independent pathways are activated to ensure the survival of *C. jejuni* in the chick cecum, although which pathways are essential for colo-

nization and survival is unknown. We can speculate that in the cecum, *C. jejuni* is growing under oxygen-limited conditions, utilizing a range of electron acceptors and electron donors for respiration, exploiting specific amino acids for growth, and consuming hemin as a primary source of iron.

We have recently completed preliminary studies to investigate whether the data reported here from the newborn chick model of colonization can be repeated if 2-week-old birds with a normal flora are used as the model for colonization.

The preliminary data suggest that similar electron transport pathways are operating and similar amino acids are being utilized (data not shown). Although the current study represents the transcriptional response of *C. jejuni* within the chick cecum for a limited period of time, it clearly indicates that specific changes in electron transport and metabolic pathways enable successful colonization within this niche.

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